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Induction of Cellular Differentiation by Retinoic Acid in vitro

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Key Words
Embryonic stem cells · Differentiation · Retinoic acid · Cardiogenesis

Abstract
Cellular differentiation by the vitamin A derivative retinoic acid (RA) has been studied with undifferentiated pluripotent embryonic carcinoma (EC) and embryonic stem (ES) cells in vitro. Both cellular systems are suitable to study differentiation of various cell types, because they recapitulate early stages of mouse embryogenesis. In vivo, RA was identified as a morphogenic and teratogenic compound and furthermore as a signalling molecule influencing gene expression in a complex manner via a family of RA receptors. Here, we summarize in vitro studies with ES and EC cells in comparison to in vivo studies that have contributed to our understanding how RA influences differentiation and regulates gene expression. We demonstrate that modulation of ES cell differentiation in vitro by RA depends on the concentration and developmental stage of application which is comparable to its stage-dependent influence on embryonic development in vivo.

Abbreviations used in this paper

BMP	bone morphogenetic protein
CRABP	cellular retinoic acid binding protein
ECC	embryonic carcinoma cells
ESC	embryonic stem cells
RA	retinoic acid
RAR	retinoic acid receptor
RXR	retinoid X receptor

Introduction

A prerequisite to study cellular differentiation in vitro is the availability of model systems that closely resemble development in vivo. Mouse embryonic stem (ES) cells, derived from the inner cell mass of mouse blastocysts, are undifferentiated, pluripotent cells that have the capacity to differentiate into cell types of all three primary germ layers [Doetschman et al., 1985]. In vivo, ES cells have therefore been used for gene targeting experiments to generate genetically altered mice [Thomas and Capecchi, 1987]. In vitro, ES cells spontaneously differentiate into many cell types and were used as a model system to study differentiation of cardiac [Wobus et al., 1991; Maltsev et al., 1993, 1994; Wobus and Guan, 1998], myogenic [Rohwedel et al., 1994, 1995, 1998b], hematopoietic [Schmitt et al., 1991; Wiles, 1993], epithelial [Bagutti et al., 1996], neuronal [Bain et al., 1995; Fraichard et al., 1995; Strübing et al., 1995; Okabe et al., 1996], vascular smooth muscle [Drab et al., 1997] and adipogenic [Dani et al.,

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1997] cells (fig. 1). In contrast, undifferentiated teratocarcinoma-derived embryonic carcinoma (EC) cells fail to differentiate into germ cells in vivo. Furthermore, EC cells have to be induced to differentiate in vitro.

During ES cell differentiation, tissue-specific genes, proteins, ion channels and receptors are differentially expressed in a pattern closely resembling the pattern observed during mouse embryogenesis [reviewed by Guan et al., 1999; see fig. 1]. This model system enables one to study modulation of differentiation by 'gain-of-function' and 'loss-of-function' approaches and the influence of exogenous factors on differentiation such as differentiation factors, growth factors, signalling molecules and extracellular matrix proteins [e.g. Simon et al., 1992; Braun and Arnold, 1994; Rohwedel et al., 1994; Wobus et al., 1994b; Rohwedel et al., 1995; Soudais et al., 1995; Fässler et al., 1996; Wobus et al., 1997; Rohwedel et al., 1998a]. With this system, we studied the influence on differentiation of one of the most important differentiation-inducing factors, the vitamin A derivative retinoic acid (RA).

Since the beginning of this century vitamin A has been known to be an important nutritional compound that particularly plays an important physiological role during the visual cycle [reviewed by Wolf, 1996]. Vitamin A deprivation during pregnancy or the early postnatal phase results in embryonic malformations [Wilson and Arkany, 1949; Wilson et al., 1953]. The finding that administration of RA could prevent these malformations suggested that RA was the biologically active form of vitamin A. RA also has a teratogenic capacity since embryonic malformations were observed when an excess of retinoids was applied [Lammer et al., 1985]. More recently, it has become clear that RA functions as a hormone with important regulatory functions during embryonic development [reviewed by Morriss-Kay and Sokolova, 1996]. Evidence exists that RA is a morphogen that forms a concentration gradient along embryonic axes and affects the anteroposterior patterning of the body axis and the limbs [Kessel and Gruss, 1991; Chen et al., 1992; Chen and Solursh, 1992; Hogan et al., 1992]. In vitro, RA induces differentiation of EC cells and ES cells into specific cell types in a time- and concentration-dependent manner [Strickland and Mahdavi, 1978; Edwards et al., 1983; Jones-Villeneuve et al., 1983; Rudnicki and McBurney, 1987; Wobus et al., 1994a, b; Blank et al., 1995; Fraichard et al., 1995; Strübing et al., 1995; Wobus et al., 1997; Dani et al., 1997; Drab et al., 1997].

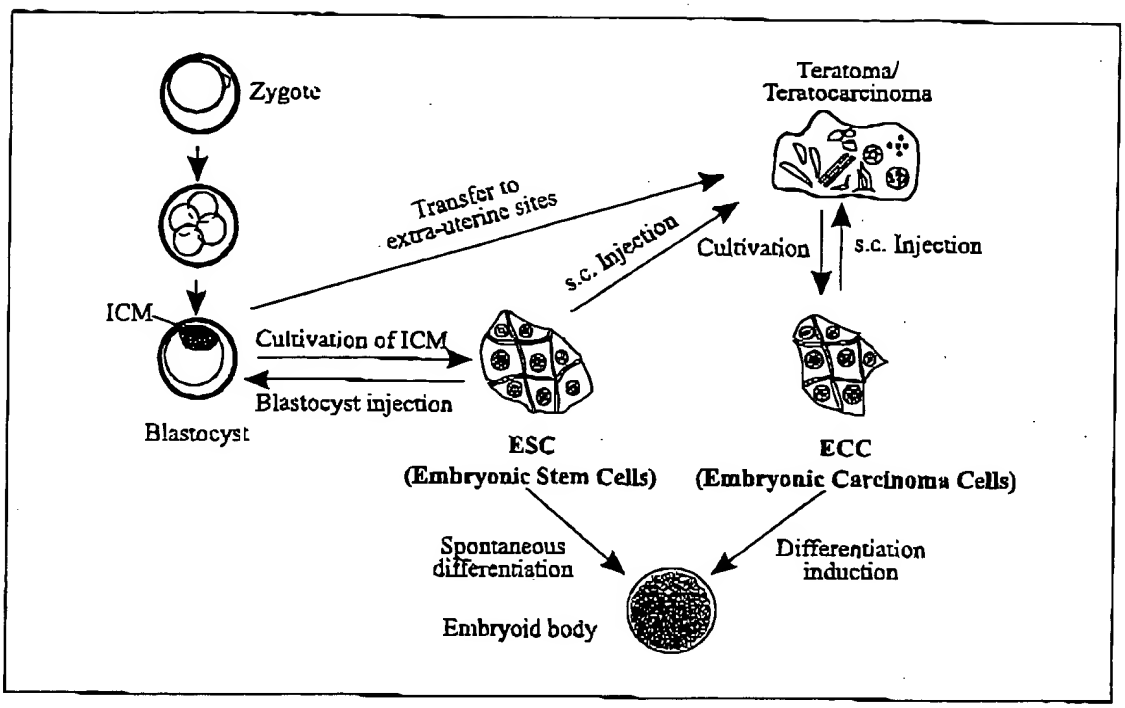
Furthermore, retinoids can influence carcinogenesis. It has been shown that transformation of cells by chemical substances can be prevented by application of retinoids. In vivo, retinoids were found to inhibit tumor development in animals and humans and therefore may serve as therapeutic agents in cancer chemoprevention [reviewed by Lotan, 1996].

The diverse effects of RA on embryonic development in vivo and cellular differentiation in vitro might be explained by complex mechanisms that regulate gene expression. RA is known to influence gene expression via two families of nuclear RA receptors, the retinoic acid receptors (RARs) and the retinoid X (9-*cis* RA) receptors (RXRs). Numerous isoforms of these receptors exist that bind to response elements of RA target genes [reviewed by Chambon, 1996].

Here, we summarize the teratogenic and morphogenic effects of RA in vivo, and the effects of RA on EC and ES cell differentiation in vitro. Furthermore, we briefly discuss the differentiation-inducing and antitumorigenic effects of RA and its molecular mechanism of action.

Teratogenic Effects of RA

Application of RA during embryogenesis resulted mainly in severe defects of craniofacial structures including the central nervous system [Lammer et al., 1985; Durston et al., 1989], the limbs [Kochhar, 1973; Alles and Sulik, 1989; Eichele, 1989; Tabin, 1991] and the axial skeleton [Sive et al., 1990; Kessel and Gruss, 1991; Kessel, 1992]. From 154 human females exposed to isotretinoin during pregnancy only a minority (~17%) had normal infants whereas most (~69%) had abortions and 14% of the newborns showed malformations [Lammer et al., 1985]. Malformations were observed in the central nervous system (86%), in craniofacial structures (81%), in the heart (57%) and in the thymus (33%). In mice, the skeletal malformations were not restricted to carniofacial structures but extended to the vertebrae and especially the limbs [Kochhar, 1973]. The multiple teratogenic effects of RA were strongly dependent on the concentration and time of application. For example, treatment of mouse pregnant females with all-*trans* RA at day 10 of gestation resulted in a complex pattern of malformations including craniofacial structures and the loss of vertebrae, whereas treatment between day 12 and 14 induced primarily limb defects [Kochhar, 1973]. Since most of the affected structures are derived from the neural tube and its derivatives, such as the neural crest cells, one might speculate that these first visible axial structures are most sensitive to RA.



Tissue-specific gene expression during ESC-derived embryoid body differentiation

Time of embryoid body differentiation	Mesoderm		Neuro-ectoderm		Cardiac muscle						Skeletal muscle							Neuronal cells						Epithelial cells				Vascular smooth muscle		
	T	BMP-4	Pax-6	Mash-1	Nkx2.5	α1CaCh	α-MHC	β-MHC	ANF	MLC-2V	Myf5	Myogenin	MyoD	Myf6	M-cadherin	γ-nAChR	ε-nAChR	Synaptophysin	NFL	NFM	Wnt-1	NFH	Neurocan	K18	K14	K10	Involucrin	V-SM-MHC-A	A II-R	SM-α-Actin
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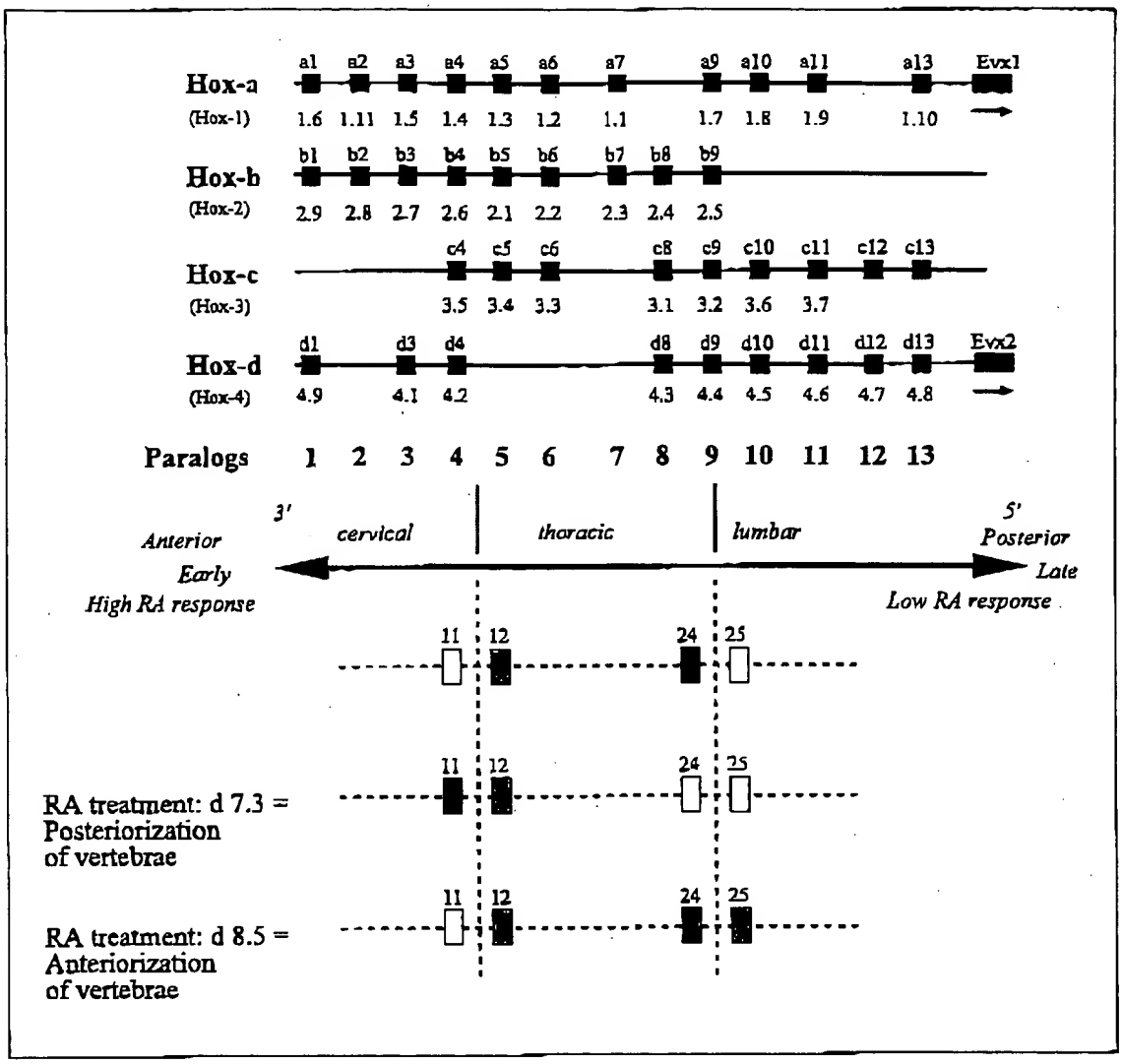


Fig. 1. ES cell differentiation in vitro recapitulates early embryonic development in vivo. ES cell-derived embryoid bodies differentially express genes characteristic for early mesodermal *(T, BMP-4)* and neuroectodermal development *(Pax-6, Mash-1)* and for cardiac *(Nkx2.5, α1CaCh, α-MHC, β-MHC, ANF* and *MLC-2V)*, myogenic *(Myf5, Myogenin, MyoD, Myf6, M-cadherin, γ-nAChR* and *ε-nAChR)*, neuronal *(NFL, NFM, Wnt-1, NFH, Synaptophysin, Neurocan)*, epithelial (keratins *K18, K14, K10* and *involucrin)* and vascular smooth muscle cell differentiation *(V-SM-MHC-A, AII-R, SM-α-Actin)* in a pattern closely resembling the pattern observed during mouse embryogenesis. s.c. = Subcutaneous; ICM = inner cell mass; T = Brachyury; BMP-4 = bone morphogenic protein 4; α1CaCh = α_1 subunit of the L-type calcium channel; MHC = myosin heavy chain; ANF = atrial natriuretic factor; MLC = myosin light chain; nAChR = nicotinic acetylcholine receptor; NFL = 68-kDa neurofilament protein; NFM = 160-kDa neurofilament protein; NFH = 200-kDa neurofilament protein; V-SM-MHC = vascular smooth muscle myosin heavy chain; A II-R = angiotensin II receptor; SM = smooth muscle [data from Maltsev et al., 1993, 1994; Rohwedel et al., 1994; Rose et al., 1994; Strübing et al., 1995; Bagutti et al., 1996; Drab et al., 1997; Wobus et al., 1997; Rohwedel et al., 1998a, b].

Fig. 2. RA modulates *Hox* gene expression and axis determination. *Hox* genes (filled boxes), assigned to 13 paralogous groups based on their location and sequence homology, are depicted in the upper panel in comparison to the colinearity of their spatiotemporally expression along the anteroposterior axis and their response to RA. Homeotic transformations that result either in posteriorization or anteriorization of vertebrae are caused by application of RA to pregnant female mice at day 7.3 or 8.5 of gestation, respectively, as schematically demonstrated for vertebrae 11, 12, 24 and 25 in the lower panel. The phenotypes of the last cervical and first lumbar vertebrae are presented as open boxes and the phenotypes of the first and last thoracic vertebrae are presented as filled boxes. After application of RA at day 7.3 the phenotype of vertebra 11 shifted from the last cervical (first line, open box 11) into the first thoracic vertebra (second line, filled box 11) and the phenotype of vertebra 24 from the last thoracic (first line, filled box 24) into the first lumbar vertebra (second line, open box 24). Thus, both 11 and 24 achieve a posterior phenotype. Vice versa, after application of RA at day 8.5 vertebra 25 changed its phenotype from the first lumbar (first line, open box 25) into the phenotype of the last thoracic vertebra (third line, filled box 25), a more anterior phenotype [modified from Krumlauf, 1994].

Influence of RA on Anteroposterior Pattern Formation in vivo

The most striking effect of RA was observed in the axis formation of *Xenopus* [Sieve et al., 1990] and mice [Kessel and Gruss, 1991; Kessel, 1992; Marshall et al., 1992] where it was accompanied by an altered expression of *Hox* genes (fig. 2). *Hox* genes encode transcription factors which contain a conserved DNA binding homeodomain [Scott et al., 1989]. They are arranged in clusters in the genome and are expressed in vertebrates along the dorsal axis in a coordinated manner, so that the 3′-located genes are expressed earlier and in the anterior region whereas the 5′-located genes are expressed later and in the posterior region (fig. 2). Thus, along the embryo axis different sets of *Hox* genes are active, specifying all the regions of the embryo including specific subsets of organs [Hunt and Krumlauf, 1991; Kessel and Gruss, 1991]. RA treatment in mice resulted in homeotic transformations of vertebrae depending on the concentration and the embryonic stage at the time of RA application. For example, application of RA at day 7.3 of gestation resulted in posterior transformations, whereas application at day 8.5 resulted in anterior transformations or agenesis of posterior segments (fig. 2). Posterior transformations were accompanied by an anterior shift of *Hox* gene expression and anterior transformations were accompanied by downregulation of *Hox* gene expression in the posterior part [Kessel and Gruss, 1991; Kessel, 1992; Krumlauf, 1994].

During chicken embryogenesis RA is synthesized by Hensen's node, a structure important for body axis induction and formation [Chen et al., 1992]. Grafting experiments have shown that the mouse and avian node can induce digit duplications in the avian wing similar to those produced by local administration of RA [Hogan et al., 1992]. In addition, at least in some cases, mice carrying 'loss-of-function' mutations in *Hox* genes showed transformations of skeletal elements along the anteroposterior axis [Le Mouellic et al., 1992; Jeannotte et al., 1993; Ramirez-Solis et al., 1993]. Since *Hox* gene expression can be induced by RA and, furthermore, RA response elements which bind RAR/RXR heterodimers have been detected in *Hox* genes [Yu et al., 1991; Pöpperl and Featherstone, 1993; Marshall et al., 1994], it seems possible that RA plays an important role in the regulation of *Hox* gene expression and thus in the specification of the body axis. However, it is also clear that embryonic axis formation is a complex process regulated by additional factors such as fibroblast growth factors, bone morphogenetic proteins (BMPs), sonic hedgehog and signalling molecules of the Wnt family [Sasai and De Robertis, 1997].

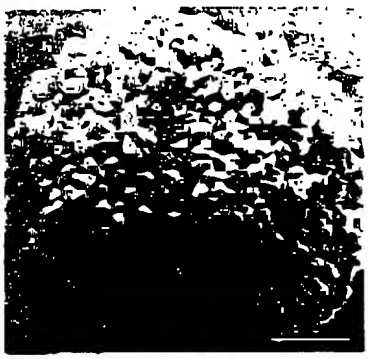
Concentration- and Time-Dependent Effects of RA on the Differentiation of EC and ES Cells in vitro

In accordance with the data obtained in vivo, RA influenced in vitro the expression of *Hox* genes in EC cells in a way that the 3′-located *Hox* genes are activated at lower concentrations of RA than the genes located towards the 5′ end [Breier et al., 1986; Simeone et al., 1990; Bonicelli et al., 1991]. In addition, developmentally regulated RA target genes were isolated from EC cells after RA-induced differentiation [Boudjelal et al., 1997]. Furthermore, similar to the in vivo situation, RA showed a concentration- and time-dependent effect on differentiation of EC and ES cells in vitro [Edwards and McBurney, 1983; Rudnicki and McBurney, 1987; Wobus et al., 1994b].

When RA was applied at a concentration of 10^{-7} to 10^{-8} *M* during the first 2 days of ES [Strübing et al., 1995] and EC [Jones-Villeneuve et al., 1983] cell-derived embryoid body development, neurogenesis was induced, whereas cardiogenesis was strongly inhibited (fig. 3). Recently, it has been shown that retinoid receptors are critical for neural posteriorization [Blumberg et al., 1997] and evidence exists that neuronal differentiation is controlled by anteroposterior patterning and requires posteriorizing signals [Papalopulu and Kintner, 1996].

In vivo this picture might be further complicated since evidence has been presented that neural induction and posteriorization are independent events [Kolm and Sive, 1997]. Neuronal induction might be caused by factors such as noggin, chordin or follistatin that inactivate BMPs [Piccolo et al., 1996; Kolm and Sieve, 1997]. The absence of BMPs could be sufficient to induce neuronal differentiation [Hemmati-Brivanlou and Melton, 1997].

With respect to inhibition of cardiac differentiation, posteriorization caused by RA may result in a lack of mesodermal precursors from which cardiomyocytes develop. In fact, cardiac cells develop from anterior lateral plate mesoderm. A block of cardiac differentiation was also reported for *Xenopus* embryos treated with RA at an early stage of development [Drysdale et al., 1997]. Furthermore, myocardial embryopathic defects were observed in malformed human infants after exposure to retinoids during pregnancy [Lammer et al., 1985]. These results suggested that induction of neuronal differentia-

EB stages	RA	NC	SMC	APC	CMC	VSMC
	0 - 2 d	++	±	–	––	±
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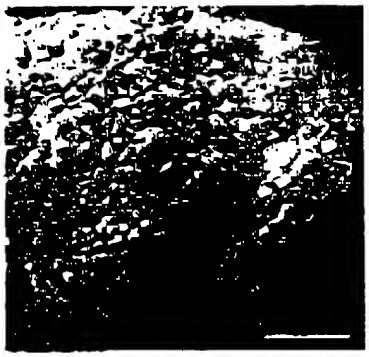
	2 - 5 d	+	++	++	––	n.d.
		10^{-8}	10^{-8}	10^{-8}		
	> 5 d	±	–	–	+	+
					10^{-8} 10^{-9}	10^{-8}

Fig. 3. Stage-specific influence of RA on ES cell differentiation. ES cells were differentiated via embryoid bodies (EBs). Scanning electron micrographs of 2-, 5- and 7-day-old embryoid bodies are shown on the left. Embryoid bodies were treated with different concentrations of all-*trans* RA during defined cultivation stages: from day 0 to 2, day 2 to 5 and after day 5. The influence of RA treatment on the differentiation efficiency of neuronal cells (NC), skeletal muscle cells (SMC), adipocytes (APC), cardiomyocytes (CMC) and vascular smooth muscle cells (VSMC) in comparison to untreated cultures is shown. ± = No effect; – = inhibition; — = strong inhibition; + = induction; ++ = strong induction. Black bars indicate induction of differentiation. Bars = 50 μm [original data: Wobus et al., 1994b, 1997; Strübing et al., 1995; Dani et al., 1997; Drab et al., 1997].

tion and inhibition of cardiogenesis may be both caused by a posteriorizing effect of RA treatment at this (early) stage of development.

Treatment of ES cell-derived embryoid bodies from day 2 to 5 with 10^{-8} *M* RA still resulted in induction of neurogenesis and inhibition of cardiogenesis. However, in addition, skeletal muscle cells [Wobus et al., 1994b] and adipocytes [Dani et al., 1997] were now induced (fig. 3). A time-dependent induction of skeletal muscle cells was also described for EC cells [Edwards and McBurney, 1983; Edwards et al., 1983; Rudnicki and McBurney, 1987]. Skeletal muscle cells and adipocytes originate from the dorsal somatic mesoderm and probably share the same mesodermal stem cell precursors [Taylor and Jones, 1979], whereas cardiomyocytes are derived from lateral plate mesoderm. Induction of skeletal muscle cell differentiation and inhibition of cardiomyocyte differentiation at the same time indicates that a developmental switch may take place at this stage. Inhibition of cardiomyocyte differentiation can be obtained by treatment of embryoid bodies with RA between day 0 and 2 and between day 2 and 5. Indeed, during these stages of embryoid body differentiation expression of mesodermal marker genes such as Brachyury and BMP-4 showed a maximum [Rohwedel et al., 1998a], suggesting that mesodermal specification takes place.

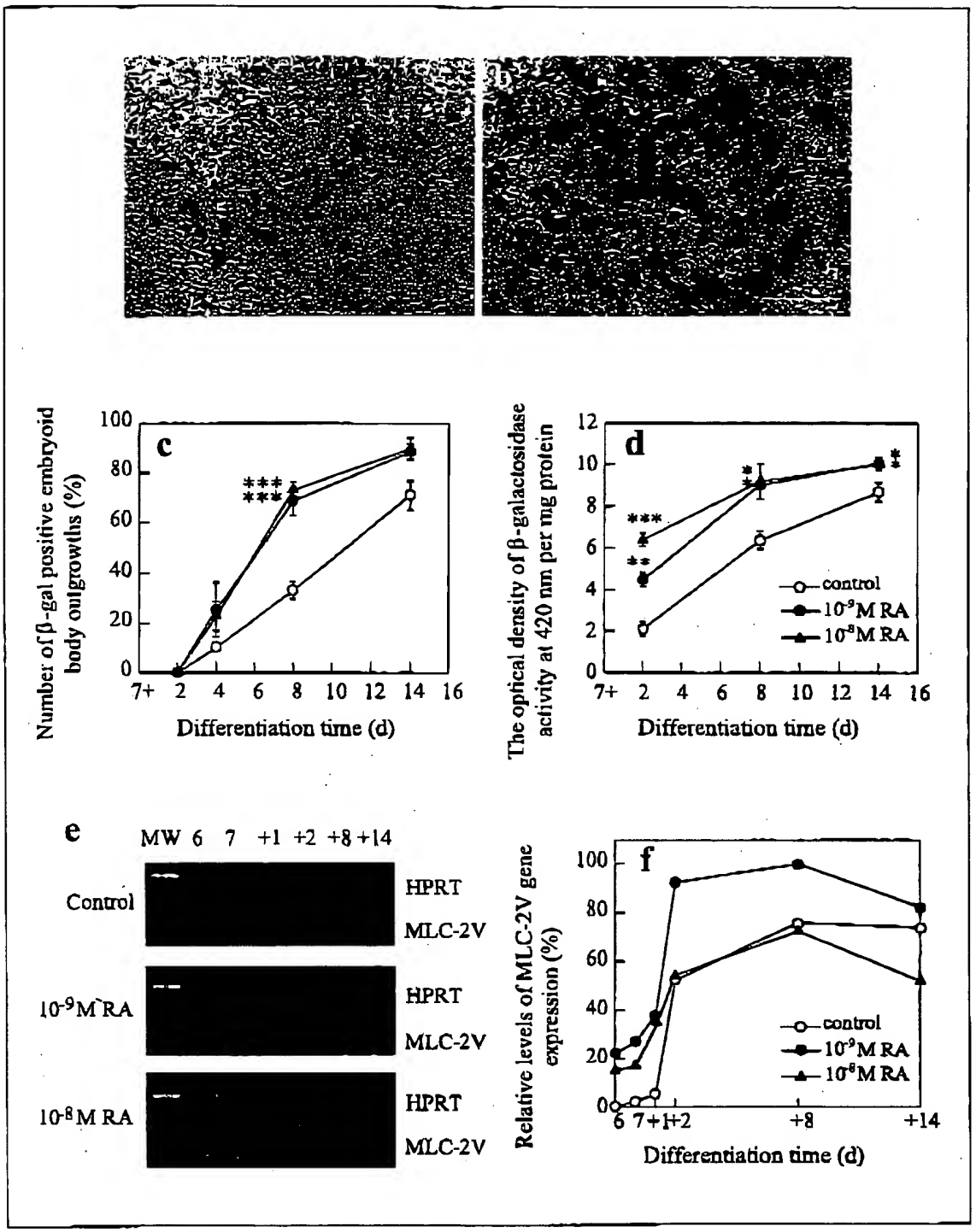
When embryoid bodies were treated with 10^{-8} *M* RA after day 5, myogenic and adipogenic differentiation was found to be inhibited, but differentiation of cardiac [Wobus et al., 1997] and of vascular smooth muscle cells [Drab et al., 1997] was induced (fig. 3). Similarly, EC cells have been induced to differentiate into vascular smooth muscle cells [Blank et al., 1995]. Induction of cardiac differentiation was even more prominent after treatment of embryoid bodies with 10^{-9} *M* RA (fig. 3). Thus, RA treatment after day 5 of embryoid body development resulted in an opposite effect compared to treatment between day 2 and 5. Similarly, in vivo, treatment of mouse embryos at day 8.5 (in comparison to day 7.3) resulted in an anteriorizing instead of a posteriorizing effect on axis formation (fig. 2).

It had already been reported more than 40 years ago that vitamin A deficiency in vivo resulted in abnormal development of the ventricular chamber of the heart [Wilson and Warkany, 1949]. In vitro, ES cell-derived cardiomyocytes undergo specialization into atrial-, ventricular-, sinusnodal- and Purkinje-like cells [reviewed by Wobus and Guan, 1998]. Therefore, we asked the question of what type of specialized cardiac cells was induced by RA. Expression of the ventricle-specific gene MLC-2V was found to be upregulated during RA-induced cardiogenic ES cell differentiation (fig. 4). Upregulation of this gene and an increase of the amount of ventricle cells [Wobus et al., 1997] indicated a specific effect of RA on cardiac development. In vivo, abnormal development of the ventricular chamber of the heart, very similar to the phenotype reported for vitamin A-deficiency [Wilson and Warkany, 1949], was observed in mice lacking the RXRα nuclear receptor [Kastner et al., 1994; Sucov et al., 1994]. Furthermore, ventricular defects with a milder phenotype compared to RXRα$^{-/-}$ mice were found in RARα/RARβ double-mutant mice [Lee et al., 1997]. These results demonstrate that RA plays an important role during formation of the ventricular chamber. In addition to this major defect, mice deficient for RXRα and RARα/RARβ-double mutants [Gruber et al., 1996; Lee et al., 1997] showed abnormal development of the conotruncus, the atrioventricular canal and the aortic sac (fig. 5).

Induction of Differentiation and Anticarcinogenic Activity of RA

As mentioned above, RA is able to induce differentiation of teratocarcinoma-derived EC cell lines. Similarly, other tumor-derived or transformed cell lines were induced to differentiate by RA. For example, the neuroblastoma-derived cell line NB2a can be induced to form branching neurites [Shea et al., 1985], and the leukemia cell line HL-60 was differentiated into granulocytes by treatment with RA [Bollag, 1994]. Induction of differentiation was most efficient when retinoids were combined with cytokines such as interleukines or tumor necrosis factor-α [Lotan, 1980; Bollag, 1994]. During the last years,

Fig. 4. RA treatment resulted in enhanced development of ventricle-like cells during ES cell-derived cardiac differentiation. Enhanced activity of the reporter construct pGNA/MLC2.1 containing a ventricle-specific promoter and the *lacZ* gene (**a–d**) and enhanced expression of the endogenous *MLC-2V* gene encoding the ventricular myosin light chain-2 isoform (**e, f**) during differentiation of a stably transfected ES cell clone after treatment with 10^{-9} and 10^{-8} *M* all-*trans* RA in comparison to (untreated) control cells. Treatment of embryoid bodies (EBs) with 10^{-9} *M* all-*trans* RA between days 5 and 15 resulted in an increase of β-galactosidase-positive cells in EB outgrowths (**b**) compared to untreated control cells (**a**) at day 15. The percentage of β-galactosidase-positive EBs (**c**) and the β-galactosidase activity in relation to cellular protein content (**d**) was evalu-



ated in control and RA-treated variants (10^{-9} and 10^{-8} *M* all-*trans* RA). Each data point represents the mean value ± standard error of the mean. Statistical significance was tested by Student's t test. Significance levels: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. The level of *MLC-2V* gene expression of 10^{-9} or 10^{-8} *M* RA-treated EBs and outgrowths of the pGNA/MLC2.1-derived ES cell clone compared to control (untreated) cells analyzed by semiquantitative RT-PCR. The ethidium bromide-stained gels of PCR products (**e**) were analyzed by computer-assisted densitometry and the data were plotted (in %) for *MLC-2V* in relation to hypoxanthine guanine phosphoribosyl-transferase *(HPRT)* gene expression, the latter used as an internal standard (**f**). Similar data were obtained in two independent experiments, and for the densitometric evaluation three independent gels were analyzed from the experiment shown in **e**. MW = Molecular weight markers. Bar (**a**, **b**) = 100 μm [**c**–**f**: original data from Wobus et al., 1997].

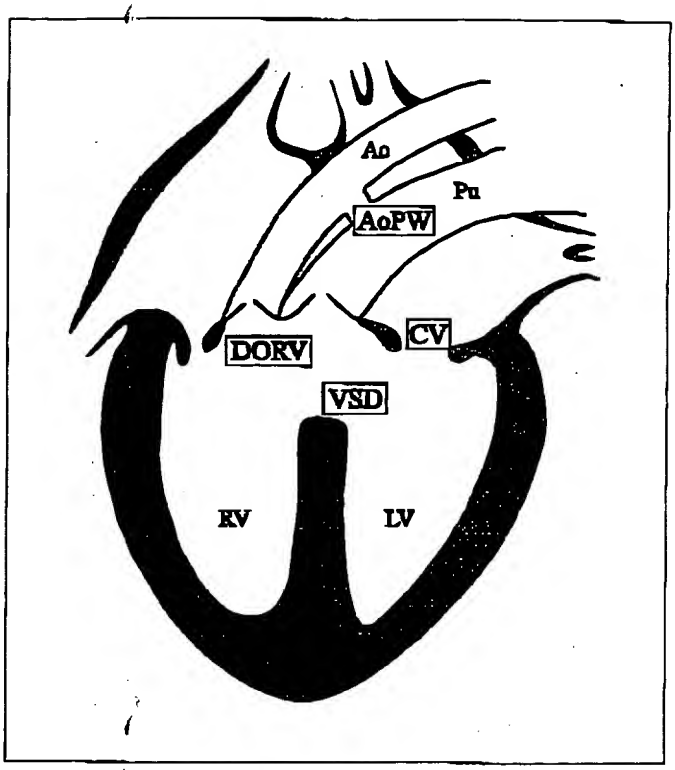


Fig. 5. Mice lacking the RXRα retinoid receptor display defects of heart development regarding mainly the ventricular chamber but also the endocardial cushions. Defects observed in hearts of mice deficient for the nuclear RA receptor RXRα and in RARα/RARβ double-mutant mice include abnormal development of (1) the ventricular chamber including ventricular septal defects (VSD), hypoplastic ventricular wall and abnormal differentiation of trabeculae and papillary muscles, (2) the aorticopulmonary septum with an aorticopulmonary window (AoPW) or persistent truncus arteriosus, (3) the conotruncus with absence of conotruncal ridges or double outlet right ventricle (DORV) and (4) the atrioventricular cushions with a common atrioventricular canal or cleft valves (CV). Ao = Aorta; Pu = pulmonalis; RV = right ventricle; LV = left ventricle [modified from Gruber et al., 1996].

all-*trans* RA has been established as a successful therapeutic agent in the treatment of acute promyelocytic leukemia in humans [Huang et al., 1988; Degos et al., 1990]. Furthermore, retinoids have been shown to prevent cancer development in head/neck and lung cancer patients [Lotan, 1996]. Inhibition of proliferation and induction of differentiation of tumor cells by RA may both rely on the ability of RA to modulate gene expression as discussed below.

Molecular Mechanisms of RA Action

The discovery of cellular RA-binding proteins (CRABP) and most importantly the nuclear RA receptors (with the isotypes RARα, β, γ and RXRα, β, γ) has significantly contributed to our understanding of how RA regulates gene expression during embryogenesis [reviewed by Mangelsdorf et al., 1995; Chambon, 1996]. Inactivation of the receptor genes in mice via homologous recombination in ES cells demonstrated that the function of CRABPI and CRABPII is dispensable [Gorry et al., 1994], whereas among the nuclear receptors particularly RXRα/RAR heterodimers seem to be essential for RA signalling [Chambon, 1996; Kastner et al., 1997]. Phenotypic alterations in mice including almost all of the abnormalities observed after postnatal vitamin A deficiency were observed in RAR mutants [Kastner et al., 1995]. The defects regarding heart development in RXRα-deficient mice have been discussed above. The following model for RA signalling is proposed (fig. 6): RA binds to CRABP that interact with the nuclear RA receptors. All-*trans* RA binds to RAR and 9-*cis* RA to RXR and RAR nuclear receptors. Homodimers of RXR or RAR/RXR heterodimers bind via a DNA binding domain to specific sites, the so-called RA response elements. RA response elements are composed of direct repeats of the sequence PuG[G/T]TCA separated by 1, 2 or 5 basepairs, and are located upstream of the target genes. Binding of the nuclear receptors then results in transcription initiation of the target gene.

The pleiotropic effects of RA on different processes during development and differentiation can be explained by regulating gene expression via diverse combinations of RA receptors [Chambon, 1996]. A high level of complexity exists in the RA signalling pathway. Firstly, the different isoforms of RA (e.g. all-*trans* RA and 9-*cis* RA) may be combined in a regulated fashion. Secondly, the two families of RA receptors (RARs and RXRs) are composed of three isotypes (α, β and γ) with several isoforms which can be combined as different RAR/RXR heterodimers. Thirdly, an additional level of complexity is generated by the formation of functionally active heterodimers of RXRs with other nuclear receptors and by interaction of RARs and RXRs with other factors that induce or inhibit gene expression [Chambon, 1996]. Various cell types express different combinations of RA receptors. For example, in EC cells of line P19, the RA receptor RARα, β and γ genes were found to be differentially expressed during differentiation [Kruyt, et al., 1991; Jonk et al., 1992]. During the process of aggregation-mediated endodermal or mesodermal differentiation, no significant alterations

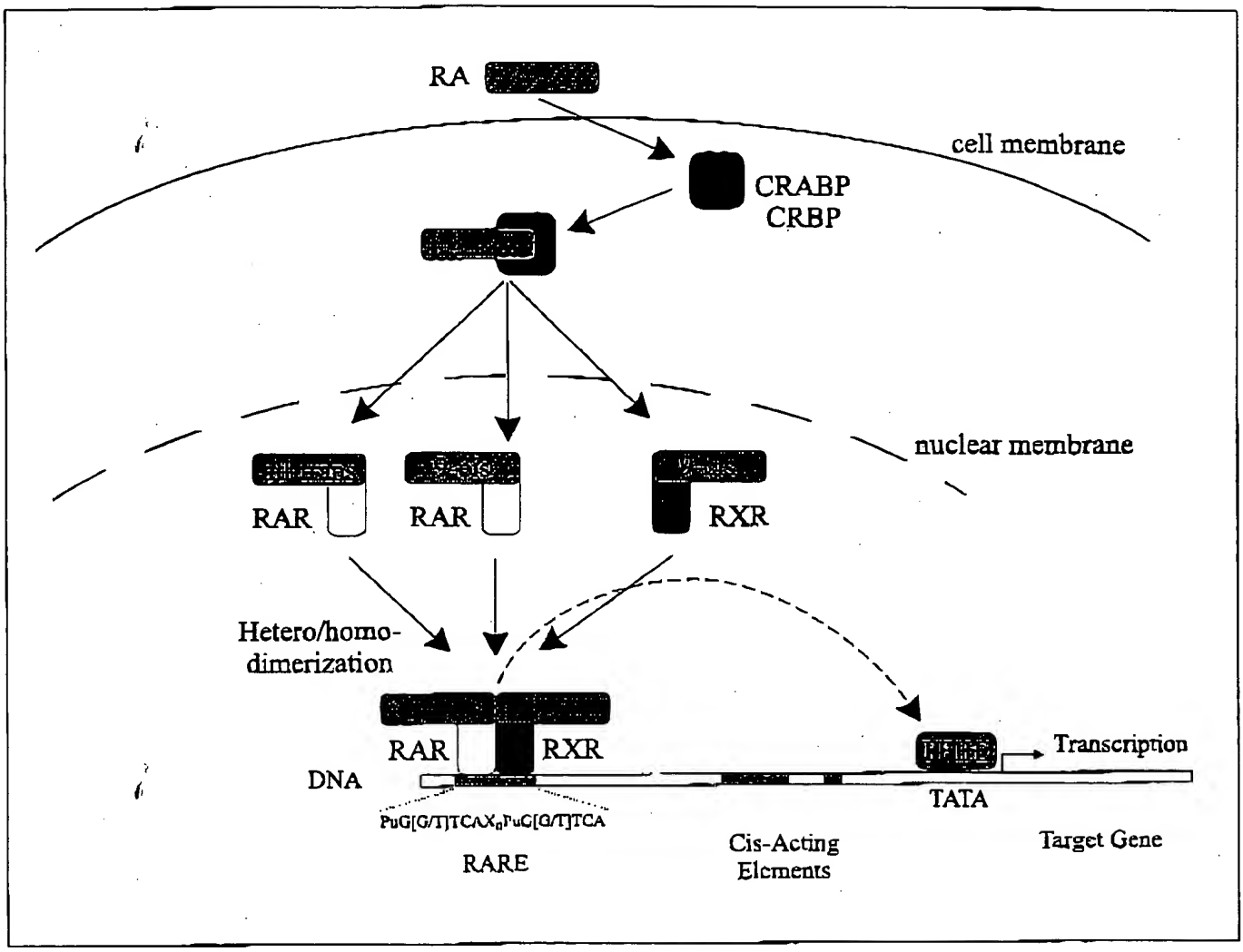


Fig. 6. Mechanism of action of RA and RA-signalling cascades. Via binding to CRABP all-*trans* RA is transferred to RAR, and 9-*cis* RA to RXR and RAR nuclear receptors. RAR/RXR heterodimers (or RXR/RXR homodimers) bind via a DNA binding domain to specific sites, the so-called RA response elements (RARE) upstream of target genes [according to Chambon, 1996].

in mRNA levels for the RA receptors RARα, β or γ could be detected. However, during neuroectodermal differentiation, a strong upregulation of RARα and β, but a rapid downregulation of RARγ mRNA was found, suggesting a role for RARα and RARβ during neuroectodermal differentiation.

As mentioned above, mice deficient for RXRα showed cardiac defects, mainly in the ventricular chamber, whereas mice with single RA receptor gene mutations in either the RARα, β, γ or RXRβ and γ genes did not show any cardiac phenotype [Lee et al., 1997]. However, when different RA receptor gene mutations were combined, specific defects were observed [Lee et al., 1997], suggesting a specific function of different receptor combinations during heart development.

RA might directly act via RA receptor on target genes that play an important role during neuroectodermal differentiation. Using a subtractive hybridization cloning strategy RA-inducible genes were isolated from EC cells of line P19 [Bouillet et al., 1995]. One of these genes, *Stra13* encoding a basic helix-loop-helix protein, is mainly expressed in neuroectoderm during mouse embryogenesis [Boudjelal et al., 1997]. Overexpression of *Stra13* in EC cells of line P19 resulted in induction of neuronal dif-

ferentiation and altered expression of mesodermal and neuronal marker genes [Boudjelal et al., 1997]. Thus, this gene is a good candidate for functioning as a repressor of mesodermal and an inducer of neuroectodermal differentiation.

With new strategies for gene isolation and identification, RA-induced EC and ES cell-derived differentiated phenotypes may help in the future to identify more RA-specific target genes. Exploring the function of such genes should help to dissect the complex RA signalling pathways and the mechanisms of action of RA on differentiation and embryonic development.

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References

Alles, A.J., K.K. Sulik (1989) Retinoic-acid-induced limb-reduction defects: Perturbation of zones of programmed cell death as a pathogenetic mechanism. Teratology *40:* 163–171.

Bagutti, C., A.M. Wobus, R. Fässler, F.M. Watt (1996) Differentiation of embryonal stem cells into keratinocytes: Comparison of wild-type and β_1 integrin-deficient cells. Dev Biol *179:* 184–196.

Bain, G., D. Kitchens, M. Yao, J.E. Huettner, D.I. Gottlieb (1995) Embryonic stem cells express neuronal properties in vitro. Dev Biol *168:* 342–357.

Blank, R.S., E.A. Swartz, M.M. Thompson, E.N. Olson, G.K. Owens (1995) A retinoic acid-induced clonal cell line derived from multipotential P19 embryonal carcinoma cells express smooth muscle characteristics. Circ Res *76:* 742–749.

Blumberg, B., J. Bolado Jr, T.A. Moreno, C. Kintner, R.M. Evans, N. Papalopulu (1997) An essential role for retinoid signaling in anteroposterior neural patterning. Development *124:* 373–379.

Bollag, W. (1994) Experimental basis of cancer combination chemotherapy with retinoids, cytokines, 1,25-dihydroxyvitamin D_3, and analogs. J Cell Biochem *56:* 427–435.

Boncinelli E, A. Simeone, D. Acampora, F. Mavilio (1991) HOX gene activation by retinoic acid. Trends Genet *7:* 329–334.

Boudjelal, M., R. Taneja, S. Matsubara, P. Bouillet, P. Dolle, P. Chambon (1997) Overexpression of Stra13, a novel retinoic acid-inducible gene of the basic helix-loop-helix family, inhibits mesodermal and promotes neuronal differentiation of P19 cells. Genes Dev *11:* 2052–2065.

Bouillet, P., M. Oulad Abdelghani, S. Vicaire, J.M. Garnier, B. Schuhbaur, P. Dolle, P. Chambon (1995) Efficient cloning of cDNAs of retinoic acid-responsive genes in P19 embryonal carcinoma cells and characterization of a novel mouse gene, Stra1 (mouse LERK-2/Eplg2). Dev Biol *170:* 420–433.

Braun, T., H.-H. Arnold (1994) ES-cells carrying two inactivated myf-5 alleles form skeletal muscle cells: Activation of an alternative myf-5-independent differentiation pathway. Dev Biol *164:* 24–36.

Breier, G., M. Bucan, U. Francke, A.M. Colberg-Poley, P. Gruss (1986) Sequential expression of murine homeo box genes during F9 EC cell differentiation. EMBO J *5:* 2209–2215.

Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors. FASEB J *10:* 940–954.

Chen, Y., L. Huang, A.F. Russo, M. Solursh (1992) Retinoic acid is enriched in Hensen's node and is developmentally regulated in the early chicken embryo. Proc Natl Acad Sci USA *89:* 10056–10059.

Chen, Y., M. Solursh (1992) Comparison of Hensen's node and retinoic acid in secondary axis induction in the early chick embryo. Dev Dyn *195:* 142–151.

Dani, C., A.G. Smith, S. Dessolin, P. Leroy, L. Staccini, P. Villageois, C. Darimont, G. Ailhaud (1997) Differentiation of embryonic stem cells into adipocytes in vitro. J Cell Sci *110:* 1279–1285.

Degos, L., C. Chomienne, M.T. Daniel, R. Berger, H. Dombret, P. Fenaux, S. Castaigne (1990) Treatment of first relapse in acute promyelocytic leukaemia with all-*trans* retinoic acid. Lancet *336:* 1440–1441.

Doetschman, T.C., H. Eistetter, M. Katz, W. Schmidt, R. Kemler (1985) The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol *87:* 27–45.

Drab, M., H. Haller, R. Bychkov, B. Erdmann, C. Lindschau, H. Haase, I. Morano, F.C. Luft, A.M. Wobus (1997) From totipotent embryonic stem cells to spontaneously contracting smooth muscle cells: A retinoic acid and db-cAMP in vitro differentiation model. FASEB J *11:* 905–915.

Drysdale, T.A., K.D. Patterson, M. Saha, P.A. Krieg (1997) Retinoic acid can block differentiation of the myocardium after heart specification. Dev Biol *188:* 205–215.

Durston, A.J., J.P. Timmermans, W.J. Hage, H.F. Hendriks, N.J. de Vries, M. Heideveld, P.D. Nieuwkoop (1989) Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature *340:* 140–144.

Edwards, M.K., J.F. Harris, M.W. McBurney (1983) Induced muscle differentiation in an embryonal carcinoma cell line. Mol Cell Biol *3:* 2280–2286.

Edwards, M.K., M.W. McBurney (1983) The concentration of retinoic acid determines the differentiated cell types formed by a teratocarcinoma cell line. Dev Biol *98:* 187–191.

Eichele, G. (1989) Retinoids and vertebrate limb pattern formation. Trends Genet *5:* 246–251.

Fässler, R., J. Rohwedel, V. Maltsev, W. Bloch, S. Lentini, K. Guan, D. Gullberg, J. Hescheler, K. Addicks, A.M. Wobus (1996) Differentiation and integrity of cardiac muscle cells are impaired in the absence of β_1 integrin. J Cell Sci *109:* 2989–2999.

Fraichard, A., O. Chassande, G. Bilbaut, C. Dehay, P. Savatier, J. Samarut (1995) In vitro differentiation of embryonic stem cells into glial cells and functional neurons. J Cell Sci *108:* 3181–3188.

Gorry, P., T. Lufkin, A. Dierich, C. Rochette-Egly, D. Decimo, P. Dolle, M. Mark, B. Durand, P. Chambon (1994) The cellular retinoic acid binding protein I is dispensable. Proc Natl Acad Sci USA *91:* 9032–9036.

Gruber, P.J., S.W. Kubalak, T. Pexieder, H.M. Sucov, R.M. Evans, K.R. Chien (1996) RXR alpha deficiency confers genetic susceptibility for aortic sac, conotruncal, atrioventricular cushion, and ventricular muscle defects in mice. J Clin Invest *98:* 1332–1343.

Guan, K., J. Rohwedel, A.M. Wobus (1999) Embryonic stem cell differentiation models: Cardiogenesis, myogenesis, neurogenesis, epithelial and vascular smooth muscle cell differentiation in vitro. Cytotechnology *30:* 211–226.

Hemmati-Brivanlou, A., D. Melton (1997) Vertebrate embryonic cells will become nerve cells unless told otherwise. Cell *88:* 13–17.

Hogan, B.L., C. Thaller, G. Eichele (1992) Evidence that Hensen's node is a site of retinoic acid synthesis. Nature *359:* 237–241.

Huang, M.E., Y.C. Ye, S.R. Chen, J.R. Chai, J.X. Lu, L. Zhoa, L.J. Gu, Z.Y. Wang (1988) Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. Blood *72:* 567–572.

Hunt, P., R. Krumlauf (1991) Deciphering the Hox code: Clues to patterning branchial regions of the head. Cell *66:* 1075–1078.

Jeannotte, L., M. Lemieux, J. Charron, F. Poirier, E.J. Robertson (1993) Specification of axial identity in the mouse: Role of the Hoxa-5 (Hox1.3) gene. Genes Dev *7:* 2085–2096.

Jones-Villeneuve, E.M., M.A. Rudnicki, J.F. Harris, M.W. McBurney (1983) Retinoic acid-induced neural differentiation of embryonal carcinoma cells. Mol Cell Biol *3:* 2271–2279.

Jouk, L.J.C., M.E.J. de Jonge, F.A. Kruyt, C.L. Mummery, P.T. van der Saag, W. Kruijer (1992) Aggregation and cell cycle dependent retinoic acid receptor mRNA expression in P19 embryonal carcinoma cells. Mech Dev *36:* 165–172.

Kastner, P., J.M. Grondona, M. Mark, A. Gansmuller, M. LeMeur, D. Decimo, J.L. Vonesch, P. Dolle, P. Chambon (1994) Genetic analysis of RXR alpha developmental function: Convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. Cell *78:* 987–1003.

Kastner, P., M. Mark, P. Chambon (1995) Nonsteroid nuclear receptors: What are genetic studies telling us about their role in real life? Cell *83:* 859–869.

Kastner, P., M. Mark, N. Ghyselinck, W. Krezel, V. Dupe, J.M. Grondona, P. Chambon (1997) Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development. Development *124:* 313–326.

Kessel, M. (1992) Respecification of vertebral identities by retinoic acid. Development *115:* 487–501.

Kessel, M., P. Gruss (1991) Homeotic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. Cell *67:* 89–104.

Kochhar, D.M. (1973) Limb development in mouse embryos. I. Analysis of teratogenic effects of retinoic acid. Teratology *7:* 289–298.

Kolm, P.J., H.L. Sive (1997) Retinoids and posterior neural induction: A reevaluation of Nieuwkoop's two-step hypothesis. Cold Spring Harb Symp Quant Biol *62:* 511–521.

Krumlauf, R. (1994) Hox genes in vertebrate development. Cell *78:* 191–201.

Kruyt, F.A.E., C.E. van den Brink, L.H.K. Defize, M.-J. Donath, P. Kastner, W. Kruijer, P. Chambon, P.T. van der Saag (1991) Transcriptional regulation of retinoic acid receptor beta in retinoic acid-sensitive and -resistant P19 embryocarcinoma cells. Mech Dev *33:* 171–178.

Lammer, E.J., D.T. Chen, R.M. Hoar, N.D. Agnish, P.J. Benke, J.T. Braun, C.J. Curry, P.M. Fernhoff, A.W. Grix Jr., I.T. Lott, J.M. Richard, S.C. Sun (1985) Retinoic acid embryopathy. N Engl J Med *313:* 837–841.

Le Mouellic, H., Y. Lallemand, P. Brulet (1992) Homeosis in the mouse induced by a null mutation in the Hox-3.1 gene. Cell *69:* 251–264.

Lee, R.Y., J. Luo, R.M. Evans, V. Giguere, H.M. Sucov (1997) Compartment-selective sensitivity of cardiovascular morphogenesis to combinations of retinoic acid receptor gene mutations. Circ Res *80:* 757–764.

Lotan, R. (1980) Effects of vitmain A and its analogs (retinoids) on normal and neoplastic cells. Biochim Biophys Acta *605:* 33–91.

Lotan, R. (1996) Retinoids in cancer chemoprevention. FASEB J *10:* 1031–1039.

Maltsev, V.A., J. Rohwedel, J. Hescheler, A.M. Wobus (1993) Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. Mech Dev *44:* 41–50.

Maltsev, V.A., A.M. Wobus, J. Rohwedel, M. Bader, J. Hescheler (1994) Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. Circ Res *75:* 233–244.

Mangelsdorf, D.J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, R.M. Evans (1995) The nuclear receptor superfamily: The second decade. Cell *83:* 835–839.

Marshall, H., S. Nonchev, M.H. Sham, I. Muchamore, A. Lumsden, R. Krumlauf (1992) Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. Nature *360:* 737–741.

Marshall, H., M. Studer, H. Popperl, S. Aparicio, A. Kuroiwa, S. Brenner, R. Krumlauf (1994) A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1. Nature *370:* 567–571.

Morriss-Kay, G.M., N. Sokolova (1996) Embryonic development and pattern formation. FASEB J *10:* 961–968.

Okabe, S., K. Forsberg-Nilsson, A.C. Spiro, M. Segal, R.D. McKay (1996) Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. Mech Dev *59:* 89–102.

Papalopulu, N., C. Kintner (1996) A posteriorising factor, retinoic acid, reveals that anteroposterior patterning controls the timing of neuronal differentiation in Xenopus neuroectoderm. Development *122:* 3409–3418.

Piccolo, S., Y. Sasai, B. Lu, E.M. De Robertis (1996) Dorsoventral patterning in Xenopus: Inhibition of ventral signals by direct binding of chordin to BMP-4. Cell *86:* 589–598.

Pöpperl, H., M.S. Featherstone (1993) Identification of a retinoic acid response element upstream of the murine Hox-4.2 gene. Mol Cell Biol *13:* 257–265.

Ramirez-Solis, R., H. Zheng, J. Whiting, R. Krumlauf, A. Bradley (1993) Hoxb-4 (Hox-2.6) mutant mice show homeotic transformation of a cervical vertebra and defects in the closure of the sternal rudiments. Cell *73:* 279–294.

Rohwedel, J., K. Guan, W. Zuschratter, S. Jin, G. Ahnert Hilger, D. Fürst, R. Fässler, A.M. Wobus (1998a) Loss of β_1 integrin function results in a retardation of myogenic, but an acceleration of neuronal, differentiation of embryonic stem cells in vitro. Dev Biol *201:* 167–184.

Rohwedel, J., V. Horák, M. Hebrok, E.M. Füchtbauer, A.M. Wobus (1995) M-twist expression inhibits mouse embryonic stem cell-derived myogenic differentiation in vitro. Exp Cell Res *220:* 92–100.

Rohwedel, J., T. Kleppisch, U. Pich, K. Guan, S. Jin, W. Zuschratter, C. Hopf, W. Hoch, J. Hescheler, V. Witzemann, A.M. Wobus (1998b) Formation of postsynaptic-like membranes during differentiation of embryonic stem cells in vitro. Exp Cell Res *239:* 214–225.

Rohwedel, J., V. Maltsev, E. Bober, H.-H. Arnold, J. Hescheler, A.M. Wobus (1994) Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: Developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. Dev Biol *164:* 87–101.

Rose, O., J. Rohwedel, S. Reinhardt, M. Bachmann, M. Cramer, M. Rotter, A. Wobus, A. Starzinski-Powitz (1994) Expression of M-cadherin protein in myogenic cells during prenatal mouse development and differentiation of embryonic stem cells in culture. Dev Dyn *201:* 245–259.

Rudnicki, M.A., M.W. McBurney (1987) Cell culture methods and induction of differentiation of embryonal carcinoma cell lines; in Robertson, E.J. (ed): Teratocarcinomas and Embryonic Stem Cells. Oxford, IRL Press, pp 19–49.

Sasai, Y., E.M. De Robertis (1997) Ectodermal patterning in vertebrate embryos. Dev Biol *182:* 5–20.

Schmitt, R.M., E. Bruyns, H.R. Snodgrass (1991) Hematopoietic development of embryonic stem cells in vitro: Cytokine and receptor gene expression. Genes Dev *5:* 728–740.

Scott, M.P., J.W. Tamkun, G.W. Hartzell (1989) The structure and function of the homeodomain. Biochim Biophys Acta *989:* 25–48.

Shea, T.B., I. Fischer, V.S. Sapirstein (1985) Effect of retinoic acid on growth and morphological differentiation of mouse NB2a neuroblastoma cells in culture. Brain Res *353:* 307–314.

Simeone, A., D. Acampora, L. Arcioni, P.W. Andrews, E. Boncinelli, F. Mavilio (1990) Sequential activation of *HOX2* homeobox genes by retinoic acid in human embryonal carcinoma cells. Nature *346:* 763–766.

Simon, M.C., L. Pevny, M.V. Wiles, G. Keller, F. Costantini, S.H. Orkin (1992) Rescue of erythroid development in gene targeted GATA-1- mouse embryonic stem cells. Nature Genet *1:* 92–98.

Sive, H.L., B.W. Draper, R.M. Harland, H. Weintraub (1990) Identification of a retinoic acid-sensitive period during primary axis formation in *Xenopus laevis*. Genes Dev *4:* 932–942.

Soudais, C., M. Bielinska, M. Heikinheimo, C.A. MacArthur, N. Narita, J.E. Saffitz, M.C. Simon, J.M. Leiden, D.B. Wilson (1955) Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro. Development *121:* 3877–3888.

Strickland, S., Mahdavi (1978) The induction of differentiation in teratocarcinoma stem cells by retinoic acid. Cell *15:* 393–403.

Strübing, C., G. Ahnert Hilger, J. Shan, B. Wiedenmann, J. Hescheler, A.M. Wobus (1995) Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. Mech Dev *53:* 275–287.

Sucov, H.M., E. Dyson, C.L. Gumeringer, J. Price, K.R. Chien, R.M. Evans (1994) RXR alpha mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. Genes Dev *8:* 1007–1018.

Tabin, C.J. (1991) Retinoids, homeoboxes, and growth factors: Toward molecular models for limb development. Cell *66:* 199–217.

Taylor, S.M., P.A. Jones (1979) Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell *17:* 771–779.

Thomas, K.R., M.R. Capecchi (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell *51:* 503–512.

Wiles, M.V. (1993) Embryonic stem cell differentiation in vitro. Methods Enzymol *225:* 900–918.

Wilson, J.G., C.B. Roth, J. Warkany (1953) An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. Am J Anat *92:* 189–217.

Wilson, J.G., J. Warkany (1949) Aortic arch and cardiac anomalies in the offspring of vitamin A deficient rats. Am J Anat *85:* 113–155.

Wobus, A.M., K. Guan (1998) Embryonic stem cell-derived cardiac differentiation: Modulation of differentiation and 'loss-of-function' analysis in vitro. Trends Cardiovasc Med *8:* 64–74.

Wobus, A.M., K. Guan, J. Shan, M.C. Wellner, J. Rohwedel, J. Guanju, B. Fleischmann, H.A. Katus, J. Hescheler, W.M. Franz (1997) Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. J Mol Cell Cardiol *29:* 1525–1539.

Wobus, A.M., T. Kleppisch, V. Maltsev, J. Hescheler (1994a) Cardiomyocyte-like cells differentiated in vitro from embryonic carcinoma cells P19 are characterized by functional expression of adrenoceptors and Ca^{2+} channels. In Vitro Cell Dev Biol Anim *30A:* 425–434.

Wobus, A.M., J. Rohwedel, V. Maltsev, J. Hescheler (1994b) In vitro differentiation of embryonic stem cells into cardiomyocytes or skeletal muscle cells is specifically modulated by retinoic acid. Rouxs Arch Dev Biol *204:* 36–45.

Wobus, A.M., G. Wallukat, J. Hescheler (1991) Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2-} channel blockers. Differentiation *48:* 173–182.

Wolf, G. (1996) A history of vitamin A and retinoids. FASEB J *10:* 1102–1107.

Yu, V.C., C. Delsert, B. Andersen, J.M. Holloway, O.V. Devary, A.M. Naar, S.Y. Kim, J.M. Boutin, C.K. Glass, M.G. Rosenfeld (1991) RXR beta: A coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell *67:* 1251–1266.